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Original Article

Amelogenin in calcified matrices of odontogenic cysts and odontogenic tumors: An immunohistochemical study

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KEYWORDS
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Abstract *Background/purpose:* There are few studies comparing the expression of enamel proteins, such as amelogenin, and cytokeratins in cyst and odontogenic tumors like in ameloblastoma and odontogenic keratocyst, indicating that amelogenin could be a potential biomarker for the aggressiveness in the odontogenic tumors. The aim of this study was to evaluate if the expression of amelogenin, cytokeratin AE1/AE3 (CKAE1/AE3) and cytokeratin 14 (CK14) in cysts and odontogenic tumors with calcified matrices such as calcifying odontogenic cyst (COC), compound (CdO) and complex (CxO) odontomas, adenomatoid odontogenic tumor (AOT) and calcifying epithelial odontogenic tumor (CEOT) as an aggressiveness indicator.
Materials and methods: Three COC, eight CxO, three CdO, twelve AOT, two CEOT and three dental germs were submitted to an immunohistochemistry panel of antibodies composed of amelogenin, CKAE1/AE3 and CK14.
Results: CKAE1/AE3 and CK14 was present in all odontogenic epithelia. The amelogenin protein was detected in prismatic and amorphous calcified matrices of epithelial origin belonging to CxO, CdO, AOT, COC and the tooth germs used as controls. On the other hand, the CEOT was the only tumor or cyst studied that did not present immunostaining for amelogenin in calcified matrices.
Conclusion: Amelogenin was detected in pathologies with a low or absent recurrence rate and excellent prognosis. CEOT was the lesion of greater clinical aggressiveness which did not express amelogenin. The presence of amelogenin in calcified matrices of odontogenic arise could be an indicator of low aggressiveness.

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Introduction

Odontogenic cysts (OC) and odontogenic tumors (OT) constitute a heterogeneous group of alterations deriving from epithelial, ectomesenchymal and mesenchymal tissue, which are part of normal tooth development. These lesions include a wide variety of different proliferative behaviors, either as hamartomas or as neoplasms.[1] Regarding the histopathological variations of these OT, some have calcified matrices similar to dental enamel, as is the case of odontomas, and others have calcified matrices with structures that are more difficult to characterize, such as the calcifications observed in the calcifying epithelial odontogenic tumor (CEOT), the adenomatoid odontogenic tumor (AOT) and calcifying odontogenic cyst (COC).[2] In the enamel matrix, the most abundant protein is amelogenin that participates in the mineralization of enamel and in cell differentiation.[3–6] This protein has been identified in some OCs and OTs.[7,8] Amelogenin is secreted by ameloblasts, which are epithelial cells that express different cytokeratins as they differentiate.[9] The expression of multiple cytokeratins in cysts and odontogenic tumors such as CK5/6, 7, 13, 14, 15, 16, 17, 19, AE1/AE3 has been reported.[10–18] CK14 is considered to be the main odontogenic epithelium cytokeratin.[7] Currently, the role of amelogenin in aggressiveness of OC and OT is controversial, since some studies find no difference in the expression of amelogenin in benign or malignant OT while other authors associate it with greater cell differentiation and less aggressiveness.[7,8,19,20]

Therefore, the present study aims to determine and compare the distribution of CKAE1/AE3, CK14 and amelogenin in odontogenic cysts and tumors with calcified matrices such as odontomas, AOT, CEOT and COC with dental germs in advanced bell stage.

Material and methods

Tissue preparation: This work is part of the Projects Fondecyt 1140905 and FIOUCh 09-11 which were approved by the ethics committee of the Faculty of Dentistry of the University of Chile. The tissues consisted of three compound odontomas, eight complex odontomas, twelve adenomatoid odontogenic tumors, two CEOT and three COC. One patient presented both lesions and they were analyzed independently. The control tissues were three dental germs surgically removed due to their close vicinity with different lesions present in the jaw bones. All of these specimens were retrieved from the files of the Institute of Oral Pathology of the Faculty of Dentistry of the University of Chile between the years 1988 and 2008.

Immunohistochemistry: The tissue sections were deparaffinized and hydrated in descending alcohols, after which they were exposed to buffer citrate 0.01 M (pH 6) in a heat source, for antigen retrieval, during 20 min. With the purpose of eliminating endogenous peroxidase activity, tissue sections were later immersed in 3% hydrogen peroxide in methanol, for a period of 10 min, at room temperature. Afterwards, tissue sections were incubated with primary antibodies during 20 min at 37 °C. The applied primary antibodies were used for amelogenin (Sigma—Aldrich, Darmstad, Germany), CKAE1/AE3 (Novocastra, Nussloch, Germany) and CK14 (Novocastra) as shown in Table 1. Later, the tissue sections were incubated with the biotinylated secondary antibody during 20 min at 37 °C. Standard streptavidin-biotin-peroxidase complex method was performed to bind the primary antibodies with the use of VECTASTAIN ® ABC. Reaction products were visualized by immersing the sections in diaminobenzidine (DAB) for 3—5 min. Nuclei were counterstained with Harris hematoxylin. For negative control studies of the antibodies, the serial sections were treated with phosphate-buffered saline, instead of primary antibodies and were confirmed to be unstained. For positive controls of antibodies for amelogenin, CK14 and CKAE1/AE3 tissues from human tooth germs, skin and oral mucosa were used respectively. All of these tissues were fixed in 10% buffered formalin, for two to four days, and then were demineralized in EDTA or Ana Morse (Formic Acid and Sodium Citrate) for 2—4 weeks and used for both histological and immunohistochemical evaluations. Serial sections of 4 μm thick were made from paraffin tissue blocks. Hematoxylin-eosin stained sections were made for routine histological examination.

Results

Of the 30 patients evaluated, 31 samples were obtained in total, since a complex odontoma (CxO) was associated with an COC. In this case, the immunohistochemical reaction was analyzed separately for the areas of CxO and COC. The

Table 1 Primary antibodies used.

Primary Antibody	Type	Dilution	Laboratory	Code
anti-amelogenin	Rabbit polyclonal	1:200	Sigma	HPA-005988
anti-cytokeratin AE1/AE3	murine monoclonal	prediluted	Novocastra	RTU-AE1/AE3
anti-cytokeratin 14	murine monoclonal	prediluted	Novocastra	RTU-LL002

study included 18 women and 13 men (Table 2), where 13 samples were located in the maxilla and 10 of them in the jaw and 8 were no-specified (Table 3).

All the selected samples are from patients in the first three decades of life (Table 2), except for the CEOT analyzed, where the patients were 54 and 73 years old.

Presence and distribution of amelogenin

The positive immunostaining for amelogenin was found in all the COC and dental germs studied, in most of the odontomas and the AOT (Fig. 1). However, it was not detected in any case of CEOT.

Regarding the distribution of amelogenin, an intense immunostaining was detected in the odontoma enamel matrix (CdO and CxO) and a slight one in reduced ameloblast epithelium, while in the AOT, the immunostaining was concentric and punctate in the eosinophilic amorphous matrix or tumor droplets. One case of AOT did not present immunostaining for amelogenin.

In the COC, the immunostaining was also concentric and homogeneous at the level of the ghost cells. In the dental germs, there was an intense immunostaining at the level of secretory ameloblasts or at the early stage of enamel apposition, whereas in the cuboidal or late stage ameloblasts, no amelogenin was detected.

Presence and distribution of cytokeratin AE1/AE3 (CKAE1/AE3)

CKAE1/AE3 was detected with intense immunostaining in all odontogenic epithelium of the samples studied (Fig. 2). In CxO and AOT, there was a marked immunoreaction in practically all strata of the epithelium, also observed in the ghost cells of the three CxOs that presented them. It was detected in ghost cells and odontogenic epithelium of all COC. In CEOT, there was an intense immunostaining of the neoplastic cells.

Table 2 Distribution according to age and gender of odontogenic tumors, calcifying odontogenic cyst and dental germs.

	Age Median Range		Female		Male		Total
	Years	Years	n	%	n	%	
CdO	13	2—14	2	66.7	1	33.3	3
CxO[a]	14.4	9—26	5	62.5	3	37.5	8
AOT	14.5	5—22	6	50.0	6	50.0	12
CEOT	63.5	54—73	1	50.0	1	50.0	2
COC[a]	14	14	2	66.7	1	33.3	3
DG	6.6	1—13	2	66.7	1	33.3	3
Total	21	2—73	18	58.1	13	41.9	31

CdO= Compound Odontoma; CxO=Complex Odontomas; AOT= Adenomatoid Odontogenic Tumor; CEOT; Calcifying Epithelial Odontogenic Tumor; COC= Calcifying Odontogenic Cyst; DG= Dental Germ.

[a] The same patient had the two lesions, but for immunohistochemical analysis, they were considered as independent lesions

Presence and distribution of cytokeratin 14 (CK14)

CK14 was detected with intense immunostaining in odontogenic epithelium of dental germs and the CEOT studied and partially in COC and in both odontoma subtypes (Fig. 3). Only one of the AOT no immunostaining was observed for CK14, which was the same case in which amelogenin was not detected.

Regarding the distribution of CK 14, it was observed in the odontogenic epithelium of tooth germs AOT, CEOT in two CdO and three CxO. In the AOT, a weak immunostaining was detected in the basal layer of the odontogenic epithelium and in the center of the duct and rosette structures. In the CEOT studied, an intense immunoreaction of this antibody was observed in practically all the neoplastic epithelium. In the COC, an immunohistochemical reaction was detected for CK14 in the odontogenic epithelium, being completely absent in the cytoplasm of the ghost cells present in both CxO and COC.

Discussion

The cyst and odontogenic tumors analyzed have mineralized structures with different degrees of similarity with the normal tissues of the developing tooth.[1,21] The association of COC with odontomas has been previously reported, with a range of 22%—75% of cases.[22,23]

In the present study, an intense immunohistochemical reaction was detected for amelogenin in the enamel matrix of dental germs in the advanced bell stage, being similar to the study of Saku et al. and Papagerakis et al. which were positive in secretory ameloblasts in the same stage.[21,24] The amelogenin in the enamel matrix showed two distribution patterns that we attribute to the early apposition stage (columnar ameloblasts), observing the amelogenin throughout all enamel matrix, while in the late stage of enamel apposition (cuboidal ameloblasts) amelogenin was not observed. That was related with the last stage of enamel maturation and protein degradation, mainly amelogenin.[25]

Table 3 Distribution according to location of odontogenic tumors, calcifying odontogenic cyst and dental germs.

	Mandible		Maxilla		No-specified (NE)		Total
	n	%	n	%	n	%	
CdO	1	33.3	0	0.0	2	66.7	3
CxO[a]	1	12.5	3	37.5	4	50.0	8
TOA	5	41.7	6	50.0	1	8.3	12
CEOT	0	0.0	1	50.0	1	50.0	2
COC[a]	1	0.0	2	66.7	0	33.3	3
DG	2	66.7	1	33.3	0	0.0	3
Total	10	32.3	13	41.9	8	25.8	31

CdO= Compound Odontoma; CxO = Complex Odontomas; AOT = Adenomatoid Odontogenic Tumor; CEOT; Calcifying Epithelial Odontogenic Tumor; COC= Calcifying Odontogenic Cyst; DG = Dental Germ.

[a] The same patient had the two lesions, but for immunohistochemical analysis, they were considered as independent lesion.

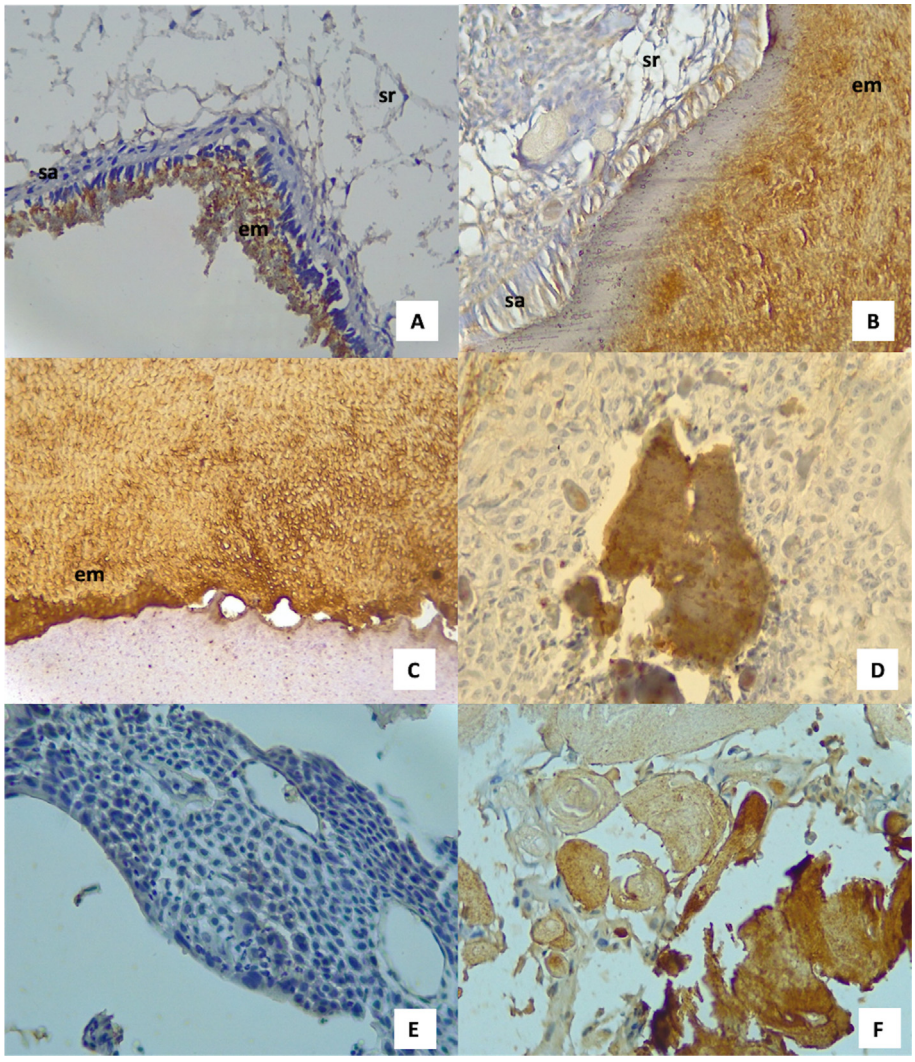

Figure 1 Amelogenin immunohistochemical staining in different odontogenic tumors (CxO,CdO, CEOT, AOT), COC and dental germ. A. Dental germ, with a positive immunostaining for enamel matrix formation, ×40 (control amelogenin); B. Complex Odontoma, with a positive immunostaining for enamel matrix formation, x40; C. Compound Odontoma, with a positive immunostaining for enamel matrix formation, x40; D. AOT, moderate immunostaining for amelogenin for the calcification indicate amelogenin presence, x40; E.CEOT, negative immunostaining for amelogenin, x40; F. COC, positive immunostaining for amelogenin in the ghost cells, x40. * sa: secretory ameloblast, em: enamel matrix, sr: stellate reticulum.

This distribution is similar to that found by Crivelini et al. who observed no immunolabeling in the reduced ameloblast epithelium and by Takata et al. who detected another enamel protein, enamelisin, in the enamel matrix of dental germs, describing that immunolabeling was expressed in zones of enamel matrix near dentin and not adjacent to dentin.[7,26] Kumamoto et al. detected amelogenin in the enamel matrix and in cells of the internal epithelium of the enamel organ of tooth germs, and they described that this expression was weaker in the stellate reticulum and the intermediate stratum.[19]

Regarding the odontogenic tumors in both types of odontomas, an intense immunohistochemical reaction was detected for amelogenin in the enamel matrix and in two CxO ghost cells, similar to Crivelini et al. results.[7] The reduced odontogenic epithelium presented a slight immunostaining, different from Mori et al. results.[27] In the study performed by Papagerakis et al., the presence of amelogenin was detected in the enamel matrix of odontomas and of the epithelial cells present.[24] Takata et al. detected enamelisin in the immature enamel present in odontomas and showed an immunostaining for enamelisin similar to the ghost cells of the COC,[26] so there are several amelogenesis-associated proteins that may be present in the enamel matrix of odontomas.

In all COC cases of this study, a marked immunoreaction for amelogenin was observed in ghost cells, presenting a concentric or homogenous distribution, similar to the results in the study by Yoshida et al. who detected amelogenin in all the ghost cells of the COC observed and only in 30% of the epithelial cells near ghost cells.[22] On the other hand, Saku et al. described that small mineralized areas immersed in the COC epithelium and superficial layer cells adjacent to the cystic cavity show a diffuse immunoreaction for amelogenin.[21]

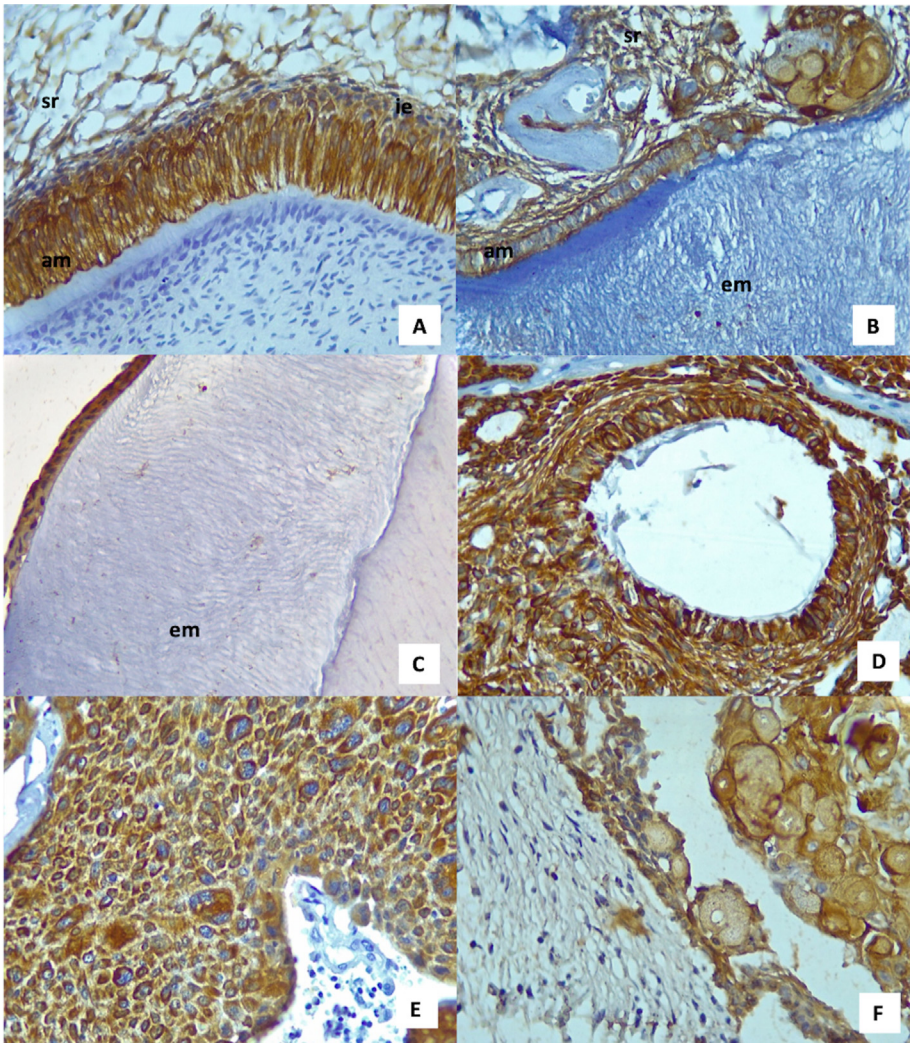

Figure 2 Cytokeratin AE1/AE3 immunohistochemical staining in different odontogenic tumors (CxO, CEOT, AOT), COC and dental germ. A. Dental germ, with a positive immunostaining for epithelial cell (ameloblast),x40.; B. Complex Odontoma, with a positive immunostaining for epithelial cells (reduced epithelium of enamel organ), x40.; C. Compound Odontoma, with a positive immunostaining for epithelial cells (reduced epithelium of enamel organ), x40; D. AOT, positive immunostaining for epithelial component (rosette, duct-type structure), x40; E. CEOT, positive immunostaining for epithelial cells, x40; F. COC, positive immunostaining for the epithelial and ghost cells, x40. * am: ameloblast, em: enamel matrix, sr: stellate reticulum, ie: intermediate epithelium.

The presence of other enamel matrix proteins such as enamelisin has been studied in odontomas and COC. These proteins had been found in ghost cells as well as in immature enamel and not in the dysplastic dentin of these lesions.[21]

In AOT, a positive immunohistochemical reaction was detected for amelogenin in areas of acellular matrix surrounded by epithelium, in the so-called "Tumor droplets" with concentric, homogeneous and punctate distribution, coinciding with others studies.[21,27] As noted by Takata et al., the formation of a typical enamel matrix with prism-like structures in AOT is very rare. Like them, we did not find the presence of prismatic enamel matrix in these tumors. The epithelial cells surrounding the "hyaline droplets" also had a moderate immunolabeling for amelogenin.[21] As in this study, Saku et al., found amelogenin in the columnar epithelial cells surrounding the ductal structures. Murata and colleagues describe an intense immunolabeling for amelogenin in the cytoplasm of columnar cells that form rosette-like structures as well as in areas called "hyaline droplets", being associated with the size of these duct-like structures.[28] Finally, Crivelini et al. did not find positive immunolabeling for amelogenin for these tumors. However, in other studies amelogenin is still a controversial biomarker for AOT.[7]

In the CEOT analyzed, the immunolabeling of amelogenin was negative both in homogeneous hyaline structures adjacent to the epithelium and in the neoplasic epithelium. Kumamoto et al. pointed out that amelogenin was expressed diffusely in neoplasic cells and did not observe immunolabeling for amelogenin in calcified areas.[19] In addition, the lack of a reaction for amelogenin in well-calcified material, suggesting only minimal amounts or absence of amelogenin, as seen in well-calcified enamel.[27] On the other hand, Saku et al. detected an intense immunolabeling of amelogenin in mineralized foci surrounded by neoplastic epithelial cells.[21] Currently, has been proposed that the expression of this

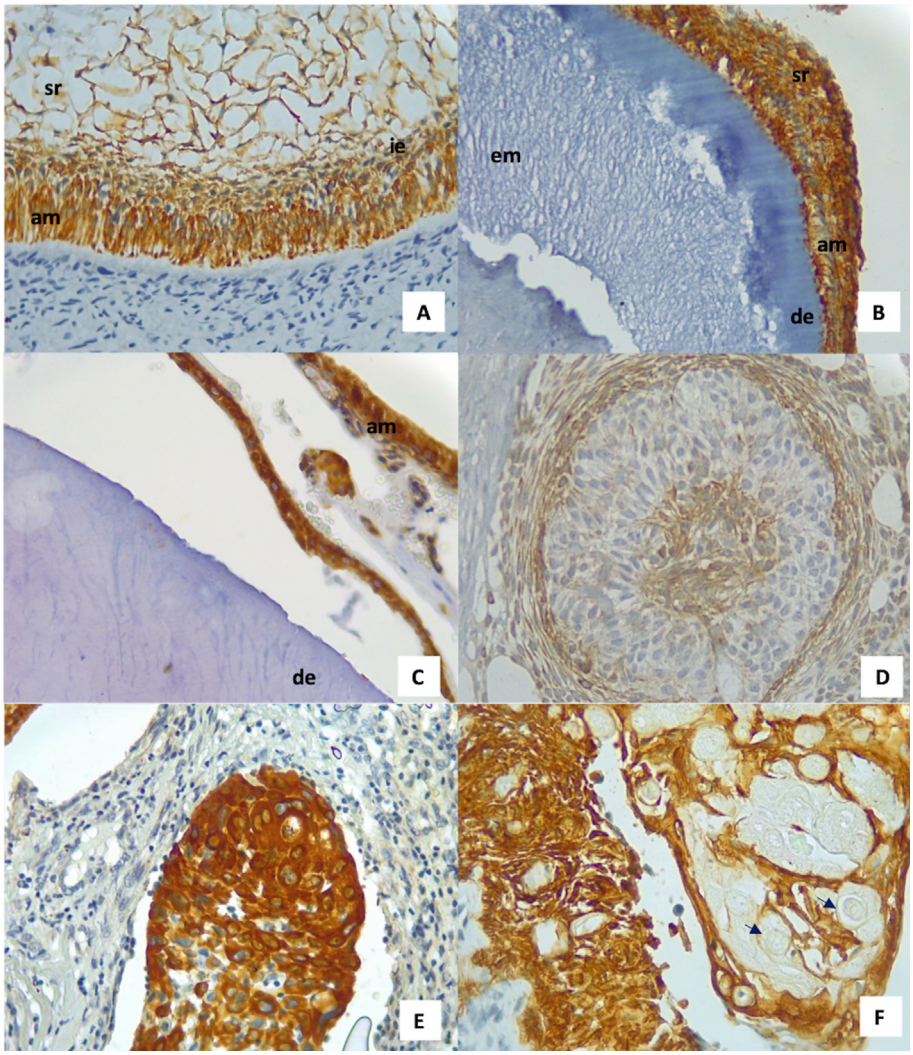

Figure 3 Cytokeratin 14 immunohistochemical staining in different odontogenic tumors (CxO, CEOT, AOT), COC and dental germ. A. Dental germ, with a positive immunostaining for epithelial cells, x40.; B. Complex Odontoma, with a positive immunostaining for epithelial cells, x40.; C. Compound Odontoma, with a positive immunostaining for ameloblastic epithelium, x40; D. AOT, mild positive immunostaining for basal epithelial cells of rossete and duct-type structure, x40; E. CEOT, positive immunostaining for CK14 in the epithelial cells, x40.; F. COC, positive immunostaining for the epithelial cells and negative in ghost cells (arrowhead), x40. * am: ameloblasts, em: enamel matrix, sr: stellate reticulum, ie: intermediate epithelium,. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein in the odontogenic epithelium would be a potential biomarker of aggressiveness been mild to moderate intensity in ameloblastoma versus teeth germs and absence of this protein in ameloblastic carcinoma.[8,20] Furthermore, the possibility of malignant transformation of CEOT is due to dedifferentiation processes or recurrences, which affect the aggressive behavior of this lesion.[29—32]

In our study, we mainly focused on calcified matrices of odontogenic cysts and tumors, where we observe the expression of amelogenin in calcified matrices of lesions with a low aggressive behavior. The lesion of greater aggressiveness studied was the epithelial calcified odontogenic tumor, which did not present positive immunomarcation for calcified matrices or neoplastic epithelium. These results suggest that the presence of amelogenin in calcified matrices of cysts and odontogenic tumors are indicative of a highly differentiated odontogenic epithelium that simulates the functioning of mature ameloblasts with low proliferation capacity, being present in low aggressive odontogenic lesions. However, the low number of CEOT cases is a limitation of this study, so it is suggested to increase the number of cases.

The immunohistochemical reaction for cytokeratin AE1/AE3 was strongly marked in all the odontogenic epithelia analyzed in dental germs. CK14 was also observed in the odontogenic epithelium of these germs, but with a weaker immunolabeling, being similar to what Apellaniz et al. indicated in their study.[33] Crivelini et al. pointed out that the dental lamina and the enamel organ express CK14, except in advanced stages of amelogenesis and specifically in secretory ameloblasts and in stellate reticulum and intermediate stratum and other studies detected a positive

immunolabeling for cytokeratin in ameloblasts independent of the stage in which they were found.[11,21]

In the present study, in both types of odontomas there was a marked immunoreaction for CKAE1/AE3 in practically all strata of the odontogenic epithelium, also observed in the ghost cells of the three CxO that presented them. CK14 was detected in odontogenic epithelium of some odontomas and was definitely not detected in the ghost cells present in the three CxO. In contrast to this study, Crivelini et al. did not detect CK14 in the ameloblast-like secretory cells present in odontomas.[11]

The presence of CKAE1/AE3 and CK14 has been detected in basal cells of the odontogenic epithelium in the COC while in ghost cells only CKAE1/AE3 has been observed,[18] coinciding with the results of the present study.

A marked immunoreaction of CKAE1/AE3 was detected in all the strata of the odontogenic epithelium in the analyzed AOT, while a weak immunolabeling of CK14 was detected in the basal stratum of the odontogenic epithelium and in the center of the rosette-like structures and ducted-like structures. Crivelini et al. detected CK14 in the epithelial cells showing different intensities in the "tumor droplets" zones or amorphous eosinophilic material.[11]

In both CEOT analyzed there was an intense immunolabeling for CKAE1/AE3 of the neoplastic cells present and practically the same pattern of immunolabeling of CK14 was detected in the neoplastic cells of the CEOT. Similar results are reported by authors such as Saku et al. and Crivelini et al. who described that the neoplastic cells present in these tumors showed a positive immunostaining for the cytokeratin.[11,21]

Hence, CKAE3/AE1 and CK14 are present in almost all odontogenic epithelia studied and the absence of CK14 is associated with advanced stages of amelogenesis like in the ghost cell of COC.

The amelogenin protein was detected in both enamel matrices in prismatic enamel of developing teeth and odontomas, and in aprismatic amorphous matrices, of epithelial origin, in adenomatoid odontogenic tumors and in COC. All these lesions have common clinical characteristics, such as low recurrence rate and excellent prognosis. On the other hand, the CEOT was the tumor of greater clinical aggressiveness studied with a higher rate of recurrence with the possibility of malignant transformation and was the only one that did not present immunostaining for amelogenin in acellular calcified matrices. Therefore, the presence of amelogenin in calcified matrices of odontogenic origin could be an indicator of low aggressiveness in relation to clinical behavior.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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